

This Page Is Inserted by IFW Operations  
and is not a part of the Official Record

## **BEST AVAILABLE IMAGES**

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

**IMAGES ARE BEST AVAILABLE COPY.**

**As rescanning documents *will not* correct images,  
please do not report the images to the  
Image Problems Mailbox.**

**PCT**WORLD INTELLECTUAL PROPERTY ORGANIZATION  
International Bureau

## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>6</sup> :</b> <b>C12N 15/31, C07K 14/31, C12N 1/21,</b> <b>C07K 16/12, A61K 39/085</b>	<b>A2</b>	<b>(11) International Publication Number: WO 95/34655</b> <b>(43) International Publication Date: 21 December 1995 (21.12.95)</b>
<b>(21) International Application Number:</b> PCT/US95/07100 <b>(22) International Filing Date:</b> 5 June 1995 (05.06.95) <b>(30) Priority Data:</b> 08/254,968 7 June 1994 (07.06.94) US <b>(71) Applicant:</b> THE ROCKEFELLER UNIVERSITY [US/US]; 1230 York Avenue, New York, NY 10021 (US). <b>(72) Inventors:</b> CHEUNG, Ambrose; Apartment #10C, 500 East 63rd Street, New York, NY 10021 (US). FISCHETTI, Vincent, A.; 440 Jaon Court, West Hempstead, NY 11552 (US). <b>(74) Agent:</b> REA, Teresa, Stanek; Burns, Doane, Swecker & Mathis, P.O. Box 1404, Alexandria, VA 22313-1404 (US).		<b>(81) Designated States:</b> CA, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  <b>Published</b> <i>Without international search report and to be republished upon receipt of that report.</i>
<b>(54) Title:</b> SURFACE PROTEIN OF STAPHYLOCOCCUS AUREUS  <b>(57) Abstract</b>  Methods and compositions are provided for preventing Staphylococcus aureus infections associated with the use of catheters and similar devices. They are based on the neutralization of ability of surface proteins of staphylococcus to adhere to the surface of the catheters at the skin-catheter junction as the first step in the systemic invasion of the host by the organism.		

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	GB	United Kingdom	MR	Mauritania
AU	Australia	GE	Georgia	MW	Malawi
BB	Barbados	GN	Guinea	NE	Niger
BE	Belgium	GR	Greece	NL	Netherlands
BF	Burkina Faso	HU	Hungary	NO	Norway
BG	Bulgaria	IE	Ireland	NZ	New Zealand
BJ	Benin	IT	Italy	PL	Poland
BR	Brazil	JP	Japan	PT	Portugal
BY	Belarus	KE	Kenya	RO	Romania
CA	Canada	KG	Kyrgyzstan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo	KR	Republic of Korea	SE	Sweden
CH	Switzerland	KZ	Kazakhstan	SI	Slovenia
CI	Côte d'Ivoire	LI	Liechtenstein	SK	Slovakia
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CN	China	LU	Luxembourg	TD	Chad
CS	Czechoslovakia	LV	Latvia	TG	Togo
CZ	Czech Republic	MC	Monaco	TJ	Tajikistan
DE	Germany	MD	Republic of Moldova	TT	Trinidad and Tobago
DK	Denmark	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	US	United States of America
FI	Finland	MN	Mongolia	UZ	Uzbekistan
FR	France			VN	Viet Nam
GA	Gabon				

## SURFACE PROTEIN OF STAPHYLOCOCCUS AUREUS

FIELD OF THE INVENTION

This invention relates to methods and compositions for preventing infections by Staphylococcus aureus (S. aureus), especially those triggered by the use of catheters prosthetic devices and heart valve replacements. More specifically, it relates to methods and compositions useful for inhibiting the ability of a surface protein of S. aureus to implement adhesion of the organism to endothelial cells or to catheters at the skin-catherter junction thereby initiating infection.

BACKGROUND OF THE INVENTION

S. aureus is one of the most frequently encountered pathogens in infections acquired in the hospital. It accounts for 25% of all hospital acquired infections resulting from the use of catheters or similar structures. Since a great majority of patients entering a hospital require some sort of intravenous device, there is a very high probability of infection. The same type of risk applies to the use of prosthetic devices such a as hip and other joint replacements because the ability of staphylococci to adhere to such devices.

One of the initial reactions of the mammalian host to the presences of a catheter is to coat the object with fibrinogen and other matrix proteins as a prelude to the systemic reaction which is intended to expel the invasion. In a hospital setting, this provides an opportunity of infection by S. aureus which attaches

itself to the fibrinogen at the skin-catheter injunction and thereafter works its way through the skin and into the blood. Since the strains prevalent in hospitals, nursing homes and other patient care facilities are often antibiotic resistant, these types of infections are extremely serious and very difficult to contain. Accordingly, the art has expended much effort to prevent such opportunistic infections.

#### BRIEF SUMMARY OF THE INVENTION

A surface protein, and the gene which expresses it, have now been discovered. This protein enables the invading bacteria to adhere to the fibrinogen. Antibodies to fibrinogen or to the surface protein will prevent bacterial adhesion and thereby inhibit infection. The protein and segments of the protein are useful as vaccines or for the production of antibodies useful for passive protection of patients prior to the use of a catheter or equivalent device.

This invention, therefore, comprises the protein itself and segments thereof, the gene and segments thereof which produce such products, vectors for the gene and its useful segments, organisms transformed by such vectors, monoclonal and polyclonal antibodies to the protein and its useful segments, vaccines produced utilizing the protein and its segments and methods of preventing S. aureus infections utilizing such products. The invention also includes diagnostic probes utilizing the gene products described herein.

THE FIGURES

There follows a brief description of the figures.

Fig. 1 Western blot of cellular fractions of clone number 14 probes with fibrinogen followed by anti-fibrinogen antibody conjugate. The control contains the lysate of an E. coli clone with pBR322 insert in  $\gamma$  Zap. The arrows indicate a 34 kD reactive band together with an upper band which may be a dimer. Results of a duplicate blot probed with  $^{125}\text{I}$  fibrinogen were similar.

Fig. 2 Western blot (A) and silver-stained gel (B) of the periplasmic extract of clone number 14 fractionated in a fibrinogen column. The Western blot was probed with fibrinogen/anti-fibrinogen conjugate. The short arrow indicates the fibrinogen-reactive band from crude lysate positive control. The long arrow marks a 34 kD protein that reacts with fibrinogen [4th lane in (A)] and is eluted with 3 M potassium thiocyanate, but not with PBS with 0.5 M NaCl nor with acid elution.

Fig. 3 Is the complete sequence of clone 36 (C36) encoding fibrinogen reactive protein.

Fig. 4 Sequence comparison of strain DB to that of coagulases from strains 8325-4, 213 and BB with the Pileup program under the GCG package. The arrows indicate the 11 amino acid sequence that is unique to the protein. This sequence shares homology with a cell wall anchor motif found in other gram

positive wall protein. However, there is no complete identify to this motif in the Genbank. The arrows at residues 409 and 419 indicate a unique sequence segment of this protein.

The amino half (residues 59-325) of this protein is primarily helical as predicted by the Garnier analysis. Two areas (residues 58-194 and 264-297) reveal a 7 residue periodicity in which residues in position 'a' and 'd' in a heptad motif 'abcdefg' are either hydrophobic or nonpolar. This pattern is consistent with a coiled-coil conformational structure. The sequence is analyzed by the Matcher Program (22).

Fig. 5 Sequence comparison of our protein (strain DB) to that of coagulases from strains 8325-4, 213 and BB with the Pileup program under the GCC package. The arrows indicate the 11 amino acid sequence that is unique to our protein. This sequence shares homology with a cell wall anchor motif found in other gram positive cell wall protein. However, there is no complete identify to this motif in the Genbank.

The following abbreviations are employed in the description of this invention:

Strain DB	- a wild strain of <u>S. aureus</u>
N2Y broth	- a commercially available growth medium
LB	- a commercially available growth medium
IPTG	- Isopropyl-beta-D-thiogalactopyranoside
BSA	- bovine serum albumin

- X-gal - 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside
- SDS - sodium dodecyl sulfate
- NP40 - a commercially available nonionic detergent
- pBR322 - a commercially available plasmid of known structure
- pBluescript - a commercially available phagemid of known structure
- SSPE - 0.15M NaCl, 10mM NaH<sub>2</sub>PO<sub>4</sub>, 1mM EDTA pH7.4
- TSE buffer - 0.1M Tris, 20% sucrose, 5mM EDTA pH8
- PBS - phosphate buffered saline

Strain DB has been deposited at the American Type Culture Collection under the accession number \_\_\_\_\_.

The following Materials and Methods section is provided for convenience and ease of understanding of this invention.

#### MATERIALS AND METHODS

##### Bacteria, plasmids and phage

Strain DB which has been phenotypically characterized (5) was used in the construction of a genomic library. E. coli strain Sure (Stratagene) was the host cell for the  $\lambda$  Zap vector. Clones 14 and 36 were phagemids derived from fibrinogen-reactive



plaques containing the insert. A pBluescript phagemid, pAC8, which contained a protein A clone was derived from a Zap genomic library of DB.

#### Media and Antibiotics

Unless otherwise indicated, the following media were used: NZY broth and LB (23) were used for the growth of E. coli strains.

#### Preparations of affinity purified goat anti-fibrinogen conjugate

Goat anti-human fibrinogen antibody obtained commercially (Cappel, West Chester PA) was affinity-purified on a fibrinogen column as previously described (7). The fibrinogen column was prepared with glutardialdehyde activated beads (Boehringer Mannheim, Indianapolis, IN) as described in the manufacturer's insert (4). The monospecificity of the affinity purified goat anti-human fibrinogen IgG was verified by an immunoblot using purified fibrinogen and plasma as antigens (7). The protein concentration of the affinity purified antibody was determined by the BCA protein assay reagent (Pierce Chemicals, Rockeford, IL). Affinity purified goat anti-fibrinogen antibody was conjugated to bovine intestinal alkaline phosphates (Sigma, St. Louis, MO) as described by Voller (32).

#### Construction and screening of staphylococcal genomic library

A genomic library of strain DB was constructed with the Zap vector that has been digested with EcoRI and dephosphorylated (Stratagene cloning kit). DB chromosomal DNA was extracted from

lysostaphin-lysed cells as previously described (5,29). Genomic DNA was sheared with a 26 gauge syringe and subject to gel filtration on Sepharose CL2B to remove fragments smaller than 1kb. Fractions containing 4-5 kb fragments were pooled, treated with T4 polymerase to produce blunt ends, methylate with EcoRI methylase (New England Biolabs, Boston, MA), insert into the EcoRI site of the Zap vector with EcoRI linkers and packaged in vitro with Gigapack packing extracts (Statagene). Over 90% of the recombinant phages were recognized as white plaques when plated on lac<sup>-</sup> host strain Sure in the presence of IPTG and X-gal.

For the screening of fibrinogen reactive plaques, recombinant phage was incubated with the E. coli host on NZY agar (23) at 42°C for gene expression. Following transfer to duplicate nitrocellulose filters (82 mm in diameter, Schleicher & Schuell, Keene, NH), the filters were blocked with 10ml of TNT buffer (10 mM Tris with 0.15 M NaCl and 0.05% Tween 20) containing 1% BSA for 1 hour at RT and then incubated at 37°C for 3h with 25 ug of fibrinogen (Sigma #4883). This fibrinogen preparation had been further purified over a protein A sepharose column to remove contaminated IgG and was found to be essentially free of contaminants as determined by a silver-stained SDS-gel containing this protein. The filters were then washed once with TNT containing 0.1% BSA and 0.1% NP40, and twice with TNT with 0.1% BSA for 5 min. each. Affinity purified goat anti-fibrinogen antibody alkaline phosphatase conjugate diluted 1:1000 in TNT buffer with 1% BSA was then incubated with the filter for 1 hour at RT. After washing the filters twice with TNT with 0.1% BSA and 0.1% NP40 and three times with TNT with 0.1% BSA for 5 min each, fibrinogen-reactive plaques were visualized with 5-bromo-

4-chloro-3-indolyl phosphate as a substrate as described by Blake et al (1). A  $\lambda$  Zap vector with a pBR322 insert plated on E.coli or E. coli cells alone served as negative controls. Positive clones detected by fibronogen/anti-fibrinogen conjugate were confirmed by allowing plaques on duplicate filters to react with  $^{125}\text{I}$  fibrinogen. This screening procedure was similar to the method described above except that  $^{125}\text{I}$  fibrinogen (100,000 cpm) was used in place of the cold unlabeled fibrinogen. Following reaction, the nitrocellulose filters were washed twice with TNT with 0.1% BSA and 0.1% NP40 and three times with TNT with 0.1% BSA for 5 min each, and finally subjected to autoradiography. Purified plaques were isolated by rescreening each positive clone at least 4 times.

#### DNA sequencing of the fibrinogen reactive clones

By infecting the E. coli host strain simultaneously with a  $\phi$ 1 helper phage (R408) and the  $\lambda$  Zap vector containing the insert, a single strand DNA containing the pBluescript phagemid with the insert can be packaged for recirculation, thereby generating subclones in E. coli cells when plated in LB/ampicillin agar (Statagene cloning kit instructions). Plasmids for sequencing were purified from E. coli by equilibrium centrifugation in Cesium chloride-ethidium bromide gradients (23). The purity of the plasmid was confirmed by digestion with restriction enzymes (New England BioLab, Beverly, MA). By using both T3 and T7 primers flanking the insert, plasmid sequencing of clones 14 and 36 were performed with the Sequenase kit (U.S. Biochemicals, Cleveland, OH) following the manufacture's instruction (27). Additional primers were obtained for sequencing from within the insert.

### Southern blot hybridization

Southern blot hybridization was performed with random primed samples of gel-purified DNA fragments as probes (12,23). Briefly, chromosomal DNA digested with restriction enzymes was resolved on 0.7% TBE gel and transferred onto Hybond-N<sup>+</sup> membrane (Amersham, Arlington Heights, IL) (14). DNA probes were labeled with <sup>32</sup>P ( -<sup>32</sup>P deoxycytidine triphosphate, Amersham) using the random primed DNA labeling kits (Boehringer Mannheim). The membrane was then hybridized with the <sup>32</sup>P labeled DNA probe at 65°C overnight, washed twice with 2X SSPE with 0.1% SDS at RT for 10 min each followed by 1X SSPE with 0.1% SDS at 65°C for 15 min. The membrane was then subject to autoradiography with an intensifying screen at -70°C.

### Expression of fibrinogen-reactive protein of *S. aureus* in *E. coli*

One of the fibrinogen-binding clones, clone 14 was evaluated for the expression of the fibrinogen binding protein in *E. coli*. *E. coli* cells containing this clone was grown in 10ml of LB with 50 ug/ml of ampicillin at 37°C until the OD<sub>600</sub> reached 1.0. Cells were collected by centrifugation at 7,000 g for 10 min and resuspended in 1.25 ml of ice cold TSE buffer (100 mM Tris, pH 8.0, containing 20% sucrose and 5mM EDTA). Lysozyme was added to a final concentration of 0.5 mg/ml and the sample was iced for 20 min. For whole cell lysate, 0.25 ml of this suspension was removed and 7.5 ul of Triton X-100 and 50 ul of DNase solution (10 mM MgCl<sub>2</sub> with 100 ug/ml of DNase) were added. The sample was frozen (-70°C) and thawed twice.

Magnesium chloride was added to the remaining 1 ml. cell suspension (50 mM final concentration) to stabilize the spheroplasts which were then pelted at 7,000 g for 15 min. The supernatant was filtered through a 0.45  $\mu$ m Millipore membrane to obtain the periplasmic fraction.

To lyse the spheroplasts, 0.25 ml of DNase solution was added to the pellet along with 0.75 of water. The spheroplasts were aspirated vigorously several times with a pasteur pipette, frozen and thawed twice as described above. The lysate generated by this treatment was centrifuged for 49,000 g for 1 hr. The supernatant filtered through a 0.4  $\mu$ m membrane was designated cytoplasmic fraction.

The transformed E. coli has been deposited at the American Type Culture Collection under the accession number \_\_\_\_\_.

#### SDS-PAGE and immunoblot analysis

Cellular extracts (10  $\mu$ l each) were separated on 9% SDS-polyacrylamide gel slabs by the method of Laemmli (21). Prestained molecular standards (BRL, Gaithersburg, MD) were run concurrently in adjacent wells. After electrophoresis, the gel was either stained with silver (Pierce Chemicals) or transferred onto nitrocellulose (30). After transfer, the nitrocellulose filters were allowed to react with fibrinogen followed by affinity purified goat anti-fibrinogen alkaline phosphatase conjugate and reactive substrate as described for the screening of the genomic library. In some experiments, the filters were incubated with  $^{125}$ I fibrinogen (250,000 cpm), washed and autoradiographed as described above.

To detect other proteins in the cell extracts, the specific antibody diluted in blocking buffer (Tris 10mM with 0.5 M NaCl and 0.05% Tween 20, pH 8.2) was incubated with the blot for 2 hours at RT. This was followed by incubation with an appropriate alkaline phosphate conjugate for 1 hour and then processed for band visualization as previously described (3).

Partial purification of the fibrinogen-reactive protein of *S. aureus*

In an attempt to purify the fibrinogen-reactive protein from clone 14, periplasmic extracts from 4 L of culture were prepared as previously described. Briefly, cells were harvested by centrifugation (7,000 g for 20 min) and resuspended in 75 ml of TSE buffer containing 37.5 mg of lysozyme. After incubation on ice for 20 min,  $MgCl_2$  was added to a final concentration of 50 mM and spheroplasts segmented at 7,000 g for 30 min. The periplasmic fraction was aspirated from the supernatant, filtered through a 0.45  $\mu$ m membrane and immediately applied to a fibrinogen column (2.5 x 20 cm) followed by rotation at 4°C overnight. The fibrinogen column was prepared by mixing 25 mg of fibrinogen and 5 gm of glutardialdehyde beads as described (4). After collecting the fall through, the column was washed with 150 ml. of PBS followed by 150 ml of PBS with 0.5 NaCl. The fibrinogen-binding protein was then eluted by rotating the column with 10 ml of 3 M potassium thiocyanate at RT for 20 Min followed by collection. In preliminary studies, a similar elution procedure with 0.1M glycine pH 3.0 was not successful. Fractions from the column were concentrated in a Centricon 10 (Amicon, Danvers, MA) and analyzed by SDS-PAGE and immunoblots with fibrinogen as described.

### Computer analysis of sequence data

DNA protein sequence analysis, and sequence comparison with database were conducted with the Sequence Analysis Software Package from the Genetics Computer Group (University of Wisconsin, Madison, WI) (9). The deduced amino acid sequence of the putative protein was compared to a sequence database by the algorithm of Pearson and Lipman (TFASTA implementation of GenBank) (25). The fibrinogen reactive protein sequence shown in the figures has been assigned to clone 36.

### RESULTS

#### Isolation of fibrinogen reactive clones

Using the foregoing procedures, a  $\lambda$  Zap library of strain DB, was screened for clones that were reactive with fibrinogen. Of 100,000 plaques screened, three novel clones, designated 14, 30 and 36, were found to be highly reactive with both  $^{125}\text{I}$  fibrinogen and fibrinogen/antifibrinogen conjugate on immunoblots. Subclones containing the pBluescript phagemid together with the insert were subsequently generated in E. coli strain Sure. Plasmid DNA from alkaline lysis minipreps of clones 14, 30 and 36, upon digestion with EcoRI which released the inserts, revealed DNA fragments of 4.6, 3.6 and 3.2kb, respectively. Using the 4.6, 3.6 and 3.2 kb fragments as separate probes, Southern blot analysis of Eco RI digests of these clones established that they hybridized each other. These clones did not hybridize with the EcoRI fragments of pAC8, a protein A probe of DB, thus eliminating the possibility of a false positive reaction between expressed protein A gene product

and goat anti-fibrinogen antibody conjugate during the screening procedure. Further analysis indicated that clone 14 comprises about 2/3 of the mature molecule, C 36, extending into the C-terminus.

Expression studies of the fibrinogen reactive protein of S. aureus

Based on restriction analysis, clones 14 and 30 were similar. Although clone 36 contained the complete gene as determined by sequence analysis, expression of the fibrinogen reactive protein with this clone was found to be difficult. Notably, a culture of clone 14 when grown to late stationary phase (i.e.  $OD_{600mm}$  1.5) also resulted in a significantly decreased yield in fibrinogen-reactive protein. This result can be explained either by toxicity of this protein on E. coli or by increased proteolytic breakdown during stationary phase. For these reasons, the expression of the partial protein was evaluated in clone 14 that has been grown to early stationary phase ( $OD_{600mm}=1.0$ ).

Expression studies of different fractions from clone 14 with Western Blots probed with either  $^{125}I$  fibrinogen or fibrinogen/anti-fibrinogen conjugate established that the protein, which has a molecular size of 34 kD was found in the whole cell, periplasmic and membrane fraction (Fig 1). In contrast, a crude lysate of an E. coli clone which contained a pBluescript phagemid with a pBR322 insert did not react with fibrinogen (Fig.1). With some fractions (e.g. membrane), there was also a higher molecular weight band, possibly a dimer, which reacted with fibrinogen. Neither of these proteins were fusions



proteins as they were not inducible with IPTG. Additionally, these bands did not react with polyclonal and monoclonal anti-beta-galactosidase antibody (1:1000 dilution) (Boehringer Mannheim) on immunoblots. The fibrinogen reactive band also did not react with affinity purified chicken anti-protein A antibody (Accurate Chemicals, Westbury, NY), thus providing additional evidence that protein A was not cloned which, by binding to IgG, could lead to false positive results.

To confirm the binding specificity of this protein to fibrinogen, periplasmic extracts which contained fewer contaminating bands were harvested from 4 L of *E. coli* cells expressing the protein of clone 14 and applied to an affinity column with fibrinogen linked beads. The cloned proteins of interest, as analyzed by silver stain and Western blots with  $^{125}\text{I}$  fibrinogen and fibrinogen/anti-fibrinogen conjugate, was found in precolumn fractions and the 3M potassium thiocyanate eluant. However, they were not found in the fall-through, PBS with 0.5 M NaCl eluant, nor in the acid eluant (glycine pH 3.0) Fig. 2). Although the protein was not purified to homogeneity in this one step procedure (Fig. 2), these results clearly indicate the binding specificity of this protein to fibrinogen.

#### Sequence analysis of the fibrinogen reactive protein

The complete sequence of the fibrinogen protein expressed by clone 30 is shown in Fig. 3. The sequence revealed an open reading frame of 1,935 nucleotides. The sequence has a guanosine-cytosine (GC) content of 34.7%, in contrast to the 30% GC content in the staphylococcal genome (10). The higher GC content is attributable to the carboxy terminal half of the

molecule (39.7%). Putative transcription and translation signals and ribosomal binding sites are indicated in Fig. 3. The first 26 amino acids have features characteristic of a bacterial signal peptide (16). Based on the predicted cleavage site, the mature protein has a predicated size of 69,991 Da with a deduced pI of 6.5.

Analysis of the deduced amino acid sequence revealed three distinct domains in this protein. With the exception of residues 27-58, the N-terminal half (residues 58-325) of the protein is primarily helical as predicted by the Garnier analysis (15). Two areas (residues 58-194 and 264-294) within the helical portion of the molecule reveal a 7 residue periodicity in which residue in positions 'a' and d' in a heptad motif 'abcdefg' are either hydrophobic or nonpolar (Fig. 4). This finding is suggestive of a stable coiled-coil conformational structure in these areas (14.22). The second domain between residues 326 and 505 denotes a proline and glycine rich region (20%). Of the 180 residues present, there are 17 proline and 19 glycine residues. This contrasts with the N-terminal portion of the molecule in which only 3% of the residues are either proline or glycine while the remaining carboxyl portion reveals a composition of 14% proline/glycine residues. The carboxyl-terminal domain (residues 506-645) consists of 5 tandem, direct repeats of 27 amino acids each followed by 5 terminal amino acids (PRVTK). Divergence is observed mainly in the outside repeats. Conformational analysis indicated that this repeat region is nonhelical and contains mostly beta-sheets. Comparison of the protein sequence with others in the Genbank database revealed significant homology to three published S. aureus coagulases from S. aureus strains 8325-4, BB and 213 (18,19,26). With the exception of residue 7

in the primary translation product of the fibrinogen reactive protein, the N-terminal 33 amino acid residue which include the leader peptides among all four sequences were identical and therefore are likely to possess identical sequence cleavage sites (see Fig. 3). Comparing residues 1 to 422 in the fibrinogen reactive protein to coagulases in strains 8325-4, BB and 213, there are 56.2%, 73.2% and 56.2% identity, respectively. The identity between residues 423 and 645 comprising the five repeated units to homologous regions in the coagulase sequences increased markedly to 93.9%, 95.1% and 96.9% for strains 8325-4, BB and 213, respectively (Fig. 5). Like that of fibrinogen-reactive protein, the C-termini of coagulase sequences of strains 8325-4, BB and 213 are composed of repeating units of 27 homologous, but no identical, amino acids followed by the terminal sequence PRVTK (Fig. 6). However, the number of repeating units differ among strains. Although the fibrinogen reactive protein sequence displayed features that are common to the coagulase sequence, a careful comparison revealed a unique stretch of 11 amino acids between residues 409 and 419 (SVTLPSITGES) in the middle of the proline/glycine rich region (Fig. 5). Of interest is the fact that the motif LPSITGES shares homology with a cell wall anchor motif (LPXTGX) found in other gram positive surface protein (12,28). However, there is no complete identity to this heptad motif among sequences in the Genbank database.

Based on all of the foregoing, it is clear that a novel fibrinogen reactive protein of S. aureus has been cloned. This protein is both structurally and functionally different from other apparently similar proteins such as the coagulases. The protein and the gene which expresses it are illustrated in Fig.

3. The figure shows the complete clone 36 and the protein it expresses. Clone 14 runs from nucleotide 684 to nucleotide 1935 in Fig. 3. The protein expressed by clone 14 runs from amino acid residue 229 to 645. Clone 30 is substantially the same as clone 14 and expresses substantially the same protein.

Sequence analysis clearly indicates that the fibrinogen-reactive proteins of this invention shares significant homology with staphylococcal coagulases. Recent evidence by Boden and Flock also suggested that the fibrinogen binding protein of *S. aureus* may possess cross-reactivity with anti-coagulase antibody (2). However, several lines of evidence indicate that clone 36 expresses a unique fibrinogen-binding protein as do clones 14 and 30. First, expression studies of clone 14 which expresses amino acid residues 229 to 645 show that the expressed protein is necessary for fibrinogen binding. In contrast, classical coagulase has been found to complex with prothrombin to form staphylothrombin which subsequently converts fibrinogen to fibrin (10,17). Secondly, there is a unique stretch of 11 amino acids in the sequence (residues 409-419) that are not found in any of coagulase sequence described. Third, this unique amino acid sequence shares homology with a cell wall anchor motif (LPXTGX) that is found to be necessary for anchoring in a variety of gram positive surface proteins (13,28). Based on these findings, it would appear that the fibrinogen reactive protein may belong to a family of coagulase-like proteins, yet it is both structurally and functionally distinct from any of the coagulases previously described.

The isolation of fibrinogen binding protein from staphylococcal whole cell lysates with conventional chromatographic methods has been reported in two studies (11,31). However, the molecular weight (420 vs 62 kD) and the amino acid composition differs widely between the two studies. In contrast to the 62 kD protein, methionine and tyrosine, but not cysteine residues, are present in the protein of this invention. The fibrinogen reactive protein described here as expressed by clone 36 comprises predominately lysine (11.2%), threonine (9.3%) and glutamic acid (9%) while glycine (16.8%), glutamic acid (15%) and lysine (13.3%) were the abundant amino acids in the 62 kD protein (31). In addition, the deduced isoelectric point (pI-6.5) of the fibrinogen reactive protein also differs from the basic pI (about 10.2) of the 62kD protein.

Previous studies have revealed that the fibrinogen binding component of S. aureus is a cell wall constituent because it is absent in staphylococcal L form (10) and because bacterial clumping in the presence of fibrinogen is abolished upon whole cell digestion with proteinases (4). In reviewing the molecular architecture of the C-terminal region of other gram positive surface-anchored proteins, it is evident that they contain several conserved features (13,14). In the C-terminal of these proteins, a charged tail (4-7 amino acids) is usually preceded by a highly hydrophobic membrane anchor (about 16-20 amino acids), the hexamer LPXTGX, a proline-glycine rich domain and a C-terminal repeat region (14). Clearly, the C-terminal region of the fibrinogen reactive protein is different from the model described. In particular, the region preceding the stop codon lacks a charged tail and a hydrophobic membrane anchor. Instead, the five terminal amino acids (PRVTK) are preceded by five

repeats of 27 amino acid each. In addition, the region N-terminal to these repeats is a broad proline/glycine region (residues 326-505) in the middle of which is a unique sequence (LPSITGE) that shares homology with a cell wall anchor motif (LPXTGX) found in other gram positive surface proteins. Notably, this molecular architecture at the C-terminus is similar to those described for pneumococcal surface protein A (35). The amino terminal half of pneumococcal surface protein A, like that of fibrinogen reactive protein, is  $\alpha$ -helical and is consistent with an  $\alpha$ -helical coiled protein conformation. The  $\alpha$ -helical region is followed by a proline-rich domain and a repeat domain consisting of ten 20-amino-acid repeats. In addition, it also lacks a classic membrane anchor and a charged tail. In contrast to the fibrinogen reactive protein, however, there is no LPXTGE motif in the C-terminal region of pneumococcal surface protein A.

The novel proteins of this invention are surface proteins of S. aureus. No such proteins have previously been detected, isolated and characterized. They are principally characterized by their ability to bind fibrinogen. The molecular weight of the protein expressed by clone 36 is about 69,991 Da and its isoelective point 6.5. The gene which expresses this protein contains about 1935 nucleotides. Other characteristic features of the protein and of segments C14 and C30 are described above. Because of the difficulty in expressing protein from clone 36, the preferred clone of this invention is clone 36. The preferred fibrinogen binding protein is the protein expressed by this clone.

The protein of this invention, as specifically described herein, will be recognized by the skilled artisan as representative of a class of protein which may differ amongst the various strains of S. aureus, but will all be characterized as having substantially the same number of amino acid residues, the same tertiary structure and binding activity. They may differ slightly in the identity of the amino acid residues at specific positions in the protein chain. All such proteins are included within the scope of this invention.

The genes which generate the proteins of this invention may also differ slightly amongst various strains, but they all have the common characteristic of producing a protein of this invention.

The genes of this invention may be employed, as will be recognized by the skilled artisan, to produce plasmids or other vectors which, in turn are useful for transforming organisms such as E. coli to produce novel strains of this bacteria which will express the proteins of the invention.

Inhibition of the binding of S. aureus to endothelial cells is a major factor in preventing infection. Accordingly antibodies to proteins expressed by clone 36, its segments clone 14 and clone 30 and even smaller segments are important factors in controlling infection. The proteins and protein segments of this invention are therefore useful to form vaccines to inhibit S. aureus infections of mammals, including humans, by administering an amount of the selected protein which will stimulate the production of protective quantities of antibodies

to limit adhesion of S. aureus. The proteins may also be used to produce antibodies in vitro which may be employed for passive immunization.

The proteins and polypeptide or peptide segments of this invention may be obtained by any of a number of known process including the recombinant DNA techniques described above.

Polypeptide and peptides within the scope of the invention containing, for example from about 6 to 20 or more amino acid segments, may be synthesized by standard solid phase procedures with appropriate amino acids using the protection, deprotection and cleavage techniques and reagents appropriate to each specific amino acid or peptide. A combination of manual and automated (e.g., Applied Biosystem 430A) solid phase techniques can be used to synthesize the novel peptides of this invention. Although less convenient, classical methods of peptide synthesis can also be employed. For background on solid phase techniques, reference is made to Andreu, D., Merrifield, R.B., Steiner, H. and Boman, H.G., (1983) Proc. Natl. Acad. Sci USA 80, 6475-6479; Andreu, D. Merrifield, R.B., Steiner, H. and Boman, H.G., (1985) Biochemistry 24, 1683-1688; Fink, J., Boman, A., Boman, H.G., and Merrifield, R.B., (June 1989) Int. J. Peptide Protein Res. 33, 412-421; Fink, J. Merrifield, R.B., Boman, A. and Boman, H.G., (1989) J. Biol. Chem. 264-6260-6267; each of which being hereby incorporated herein by reference.

The products of the invention are amphoteric. They can exist and be utilized as free bases or as pharmaceutically acceptable metallic or acid addition salts. Suitable metallic salts include alkali and alkaline earth metal salts, preferably



sodium or potassium salts. Acid addition salts may be prepared from a wide variety of organic and inorganic acids including mineral acids, for example citric, lactic, maleic, tartaric, phosphoric and hydrochloric acids. These salts can be prepared by procedures well known to those skilled in the art.

For use as a vaccine, it is presently preferred to administer the selected product in a pharmaceutically acceptable carrier such as a buffer. Mice or other mammals, including humans, when so immunized are protected from colonization and subsequent infection by S. aureus.

Typically, the patient to be protected will be treated with product of the invention in an amount which is effective to elicit a protective immune response. The selected agent may be administered alone or in a pharmaceutically acceptable liquid or solid carrier in which it may be dispersed, dissolved or suspended. If, for example, the patient is to be treated intravenously, the peptide may be suspended as a free base or dissolved as a metallic salt in isotonic aqueous buffer. Other methods of treatment and pharmaceutically acceptable carriers will be apparent to the skilled artisan.

The proteins, polypeptides and peptides of this invention and the genes or oligonucleotides which are employed in their expression are useful as probes for genes and proteins. They are also useful to raise antibodies by which specific strains of streptococci can be identified. The sequence of nucleotides which elicit the unique segment from position 409 to position 419 and modification of this sequence are especially useful as diagnostic probes to identify S. aureus strains. The procedure

is well known to the skilled artisan for identifying other infectious organisms. It involves the preparation of labeled oligonucleotides which are used to probe the DNA released from the suspected gram positive bacteria by cell lysis, either mechanically or enzymatically.

The following citations are mentioned in the application. They are incorporated by reference.

#### CITATIONS

1. Blake, M.S., K.H. Johnston, G.J. Russell-Jones, and E.C. Gotschlich. 1984. A rapid sensitive method for detection of alkaline phosphatase-conjugated anti-body on Western blots. Anal. Biochem 136:175-179.
2. Boden, M.K. and J.I. Flock. 1989. Fibrinogen-binding protein/clumping factor from Staphylococcus aureus. Infect. Immun. 57:2358-2363.
3. Cheung, A.L. and V.A. Fischetti. 1989. The role of surface proteins in staphylococcal adherence to fibers in vitro. J. Clin. Invest. 83:2041-2049.
4. Cheung, A.L. and V.A. Fischetti. 1990. The role of fibrinogen in staphylococcal adherence to catheters in vitro. J. Infect. Dis. 161:1177-1186.
5. Cheung, A.L., J.M. Koomey, C.A. Butler, S.J. Projan, and V.A. Fischetti. 1992. Regulation of exprotein expression in Staphylococcus aureus by a locus (sar) distinct from agr. Proc. Natl. Acad. Sci. USA. 89:6462-6466.

6. Cheung, A.L., J.M. Koomey, S.Lee, E.A. Jafe, and V.A. Fischetti. 1991. Recombinant human tumor necrosis factor alpha promotes adherence of Staphylococcus aureus to endothelial cells. Infect.Immun.59:3827-3831.
7. Cheung, A.L., M.Krishnan, E.A. Jaffe, and V.A. Fischetti. 1991. Fibrinogen acts as a bridging molecule in the adherence of Staphylococcus aureus to culture human endothelial cells. J. Clin.Invest. 87:2236-2245.
8. Cohen, M.L. 1992. Epidemiology of drug resistance: implications for a post-antibiotic era. Science 257:1050-1055.
9. Devereux, J.,P. Haeberli, and O. Smithies. 1984. A comprehensive set of sequences of analysis programs for the VAX. Nucl.Acids Res. 12:387-395.
10. Easom, C.S.F. and C. Adlam. 1983. Staphylococci and staphylococcal infections, Academic Press, New York.
11. Espersen, F., I. Clemmensen, and V. Barkholt. 1985. Isolation of Staphylococcus aureus clumping factor. Infect.Immun. 49:700-708.
12. Feinberg, A.P. and B. Vogelstein. 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal.Biochem. 132:6-13.
13. Fischetti, V.A., V. Pancholi, and O. Schneewind. 1990. Conservation of a hexapeptide sequence in the anchor region of surface proteins from gram-bacteria. Mol.Microbiol. 4:1603-1605.

14. Fischetti, V.A., V. Pancholi, P. Sellers, J. Schmidt, G. Landau, X. Xu, and O. Schneewind. 1992. Streptococcal M protein: A common structural motif used by gram positive bacteria for biologically active surface molecules, p.31-38. In T.K. Korhonen, T. Hovi, and P.H. Malela (ed.), Molecular recognition in host-parasite interactions, Plenum Press, New York.
15. Garnier, J., D.J. Osguthorpe, and B. Robson. 1978. Analysis of the accuracy and implications of simple methods for predicting the secondary structure of globular proteins. *J. Mol. Biol.* 120:97-120.
16. Halvorson, H.O. and D. Perlman. 1983. A putative signal peptidase recognition site and sequence in eukaryotic and prokaryotic signal peptides. *J. Mol. Biol.* 167:391-409.
17. Hemker, H.C., M. Bas, and A.D. Muller. 1975. *Biochim Biophys. Acta* 379:180-188.
18. Kaida, S., T. Miyata, Y. Yoshizawa, H. Igarashi, and S. Iwanaga. 1989. Nucleotide and deduced amino acid sequences of staphylocoagulase gene from Staphylococcus aureus strain 213. *Nucl. Acids Res.* 17:8871.
19. Kaida, S., T. Miyata, Y. Yoshizawa, S. Kawabata, T. Morita, H. Igarashi, and S. Iwanaga. 1987. Nucleotide sequence of the staphylocoagulase gene: its unique COOH-terminal 8 tandem repeats. *J. Biochem.* 102:1177-1186.

20. Kyte, J. and R.F. Doolittle. 1982. A simple method for displaying the hydropathic character of a protein. *J.Mol.Biol.*157:105-132.
21. Laemmli, U.K. 1970. Cleavage of structure proteins during the assembly of the head of bacteriophage T4, *Nature* 227:680-685.
22. Lupas, A., M. Van Dyke, and J. Stock. 1991. Predicting coiled coils from protein sequences. *Science* 252:1162-1164.
23. Manaitis, T., E.F. Fritsch, and J. Sambrook. 1989. *Molecular cloning, a laboratory manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
24. Neu, H.C. 1992. The crisis in antibiotic resistance. *Science* 257:1064-1072.
25. Pearson, W.R. and D.L. Lipman. 1988. improved tolls for biological sequence comparison. *Proc.Natl.Acad.Sci.USA.*88:4781-4785.
26. Phonimdaeng, P., M. O'Reilly, P. Nowlan, A.J. Bramley, and T. Foster. 1990. The coagualse of Staphylococcus aureus 8325-4. Sequence analysis and virulence of site-specific coagualse-deficient mutants. *Mol.Microbiol.* 4:393-404.
27. Sanger, F., S. Nicklen, and A.R. Coulson. 1977. DNA sequencing with chain terminating inhibitors. *Proc.Natl.Acad.Sci.USA.*74:5463.

28. Schneewind, O., P. Model, and V.A. Fischetti. 1992. Sorting of protein A to the staphylococcal cell wall. *Cell* 70:267-281.
29. So, M., J.H. Crosa, and S. Falkow. 1975. Polynucleotide sequence relationships among Ent plasmids and the relationship between Ent and other plasmids. *J. Bacteriol.* 121:234-238.
30. Towbin, H., T. Staehelin, and J. Gordon. 1970. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proc. Natl. Acad. Sci. USA.* 76:4350-4354.
31. Usui, Y. 1986. Biochemical properties of fibrinogen binding (clumping factor) of the staphylococcal cell surface. *Zentralbl. Bakteriol. Mikrobiol. Hyg. [A]* 262:287-297.
32. Voller, A., D.E. Bidwell, and A. Barlett. 1976. Enzyme immunoassays in diagnostic medicine. *Bull. World Health Organ.* 53:55-65.
33. Waldvogel, F.A. 1985. *Staphylococcus aureus*, p.1097-1116. In G.L. Mandell, R.G. Jr. Douglas, and J.E. Bennett (ed.), *Principles and Practice of Infectious Diseases*, John Wiley & Sons, New York.
34. Watanakunakorn, C. and I.M. Baird. 1977. Staphylococcus aureus bacteremia and endocarditis associated with a removable infected intravenous device. *Am. J. Med.* 63:253-256.

35. Yother, J. and D.E. Briles. 1992. Structural properties and evolutionary relationship of PspA, a surface protein of Streptococcus pneumoniae, as revealed by sequence analysis J.Bacteriol. 174:601-609.



WHAT IS CLAIMED IS:

1. A gene of the group clone 14, clone 30 and clone 36 which expresses a fibrinogen binding surface protein on S. aureus and segments and analogs of said gene capable of expressing surface proteins on S. aureus having substantially the same activity.
2. A surface protein expressed by a gene of claim 1.
3. A plasmid vector carrying a gene of claim 1.
4. A microorganism transformed by a gene of claim 1 and capable of expressing a fibrinogen binding protein.
5. An antibody to a surface protein of claim 2.
6. A vaccine effective to inhibit the adhesion of S. aureus to fibrinogen because it contains a protein of claim 2.

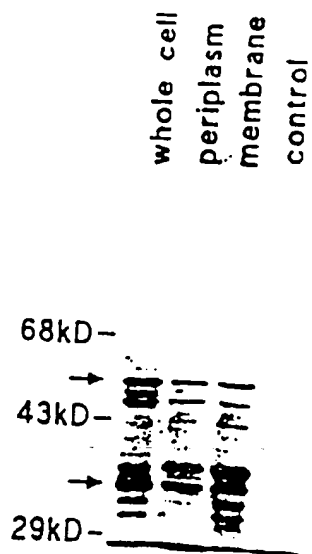


Fig. 1

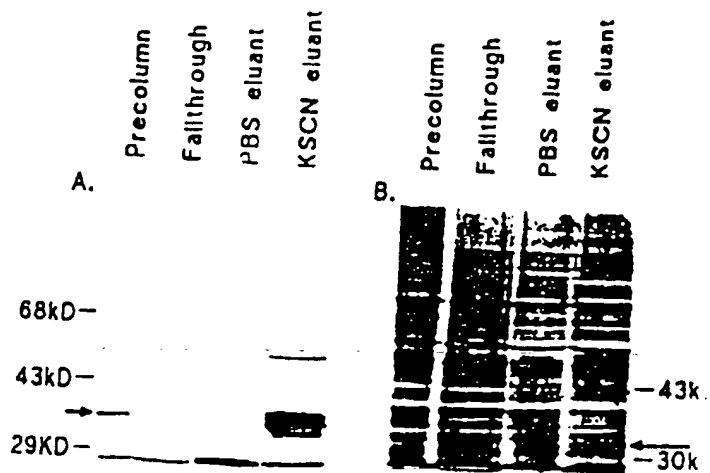


Fig. 2

10 20 30 40 50 60  
ATGAAAAAGCAAATAATATTCCTAGGCGCATTAGCAGTTGCATCTAGCTTATTTACATGG  
METLysLysGlnIleIlePheLeuGlyAlaLeuAlaValAlaSerSerLeuPheThrTrp

70 80 90 100 110 120  
GATAACAAAGCAGATGCGATAGTAACAAAGGATTATAGTGGGAAATCACAAGTTCAAAAA  
AspAsnLysAlaAspAlaIleValThrLysAspTyrSerGlyLysSerGlnValGlnLys

130 140 150 160 170 180  
AAAGAGCGCCAAAATAGTGCTTCAATATCAGATAGTTATTATTGGGATATCATTAAAAAT  
LysGluArgGlnAsnSerAlaSerIleSerAspSerTyrTyrTrpAspIleIleLysAsn

190 200 210 220 230 240  
CTAGAATTACAATTTACTGCTGCATTAGATTTATTAGAAGATTATCGATATGGTGAAAAA  
LeuGluLeuGlnPheThrAlaAlaLeuAspLeuLeuGluAspTyrArgTyrGlyGluLys

250 260 270 280 290 300  
GAGTATGAAAAGCAAAGATCAACTAATGACAAGGATATTAAGTGAAGTCAAGTATTTA  
GluTyrGluLysAlaLysAspGlnLeuMETThrArgIleLeuSerGluValLysTyrLeu

310 320 330 340 350 360  
CTTGAGCAAAAAATTAAAGAATATGACAAGTATAAAGATTTATATAAGAATATATGAGT  
LeuGluGlnLysIleLysGluTyrAspLysTyrLysAspLeuTyrLysGluTyrMETSer

370 380 390 400 410 420  
AAAAATCCAACGTCAAAGGTAAAAAGAGCAAATTTTGATCAATATAATATCGAAGACCTA  
LysAsnProThrSerLysValLysArgAlaAsnPheAspGlnTyrAsnIleGluAspLeu

430 440 450 460 470 480  
AGAGAAAAAGAATATAATGATTTACTAAGTTCTATTAAAGATGCGGTAGAAACATTTAAA  
ArgGluLysGluTyrAsnAspLeuLeuSerSerIleLysAspAlaValGluThrPheLys

490 500 510 520 530 540  
TCAGATGTACAAAAATAGAATATGAAAATAAAGAGTTAAATCTTATTCTTACGAAGAA  
SerAspValGlnLysIleGluTyrGluAsnLysGluLeuLysSerTyrSerTyrGluGlu

550 560 570 580 590 600  
GAAAAGAAGGCTGCTTCTAGAGTTGATGATTTAGCAAATAAAGCGTATAGCGTTTATTTT  
GluLysLysAlaAlaSerArgValAspAspLeuAlaAsnLysAlaTyrSerValTyrPhe

610 620 630 640 650 660  
GCGTTTGTAGGGATACACAACATAAACTGAGGCATTAGAGTTAAAGCGAAAGTGGAT  
AlaPheValArgAspThrGlnHisLysThrGluAlaLeuGluLeuLysAlaLysValAsp

670 680 690 700 710 720  
TTAGTTT TAGGTGATGAGGACAAACCACATCGCATCTCTAATAAAAGAATTGAAAATGAA  
LeuValLeuGlyAspGluAspLysProHisArgIleSerAsnLysArgIleGluAsnGlu

730 740 750 760 770 780  
ATGCTAAAAGATTTAGAATCTATTATTGAAGATTCTTTATAGAACTGGTTTAAATAAA  
METLeuLysAspLeuGluSerIleIleGluAspPhePheIleGluThrGlyLeuAsnLys

790 800 810 820 830 840  
CCTGATAATATTACGAGTTATGATAGTAGTAAACATGACTATAAACATCATAGAGAAGGC  
ProAspAsnIleThrSerTyrAspSerSerLysHisAspTyrLysHisHisArgGluGly

850 860 870 880 890 900  
TTTGAGGCTCTAGTTAAAGAAACAAGAGAAGCCGTCGATAAGGCTGACGAATCTTGGA  
PheGluAlaLeuValLysGluThrArgGluAlaValAspLysAlaAspGluSerTrpLys

910 920 930 940 950 960  
ACTAAACTGTCAAAACATATGGTGAAGCTGAAACAAAAGCACATGTTGTAAAAGAAGAG  
ThrLysThrValLysThrTyrGlyGluAlaGluThrLysAlaHisValValLysGluGlu

970 980 990 1000 1010 1020  
AAGAAAGTTGAAGAACCTCAAGCACCTAAAGTTGGAACCAAGGGGATAAACTACA  
LysLysValGluGluProGlnAlaProLysValGlyAsnGlnGlnGlyAspLysThrThr

1030 1040 1050 1060 1070 1080  
GTTGATAAAGAAGTTGAAGCAACACAACCAAGTGGCACAGCATTTAGTTGGAATTCACAG  
ValAspLysGluValGluAlaThrGlnProValAlaGlnHisLeuValGlyIleProGln

1090 1100 1110 1120 1130 1140  
GGCACAATTACAGGTGAAATTGTAAAGGTCCAGACTATCCAACGATGGAAAATAAAATG  
GlyThrIleThrGlyGluIleValLysGlyProAspTyrProThrMETGluAsnLysMET

1150 1160 1170 1180 1190 1200  
TTACAAGGTGAAATAGTTCAAGGTCCAGATTTTCTAACAAATGGAACAAAACAGACCATCT  
LeuGlnGlyGluIleValGlnGlyProAspPheLeuThrMETGluGlnAsnArgProSer

1210 1220 1230 1240 1250 1260  
TTAAGTGACAATTATACACAACCATCTGTGACTTTACCGTCAATTACAGGTGAAAGTACA  
LeuSerAspAsnTyrThrGlnProSerValThrLeuProSerIleThrGlyGluSerThr

1270 1280 1290 1300 1310 1320  
CCAACGAACCTATTTTAGAAGGTCTTGAAGGTAGCTCATCTAACTTGAAATAAAACCA  
ProThrAsnProIleLeuGluGlyLeuGluGlySerSerSerLysLeuGluIleLysPro

1330 1340 1350 1360 1370 1380  
CAAGGTACTGAATCAACGTTGAAAGGTACTCAAGGAGAATCAAGTGATATTGAAGTTAAA  
GlnGlyThrGluSerThrLeuLysGlyThrGlnGlyGluSerSerAspIleGluValLys

1390 1400 1410 1420 1430 1440  
CCTCAAGCAACTGAAACAACAGAAGCTTCTCAATATGGTCCGAGACCGCAATTTAACAAA  
ProGlnAlaThrGluThrThrGluAlaSerGlnTyrGlyProArgProGlnPheAsnLys

1450 1460 1470 1480 1490 1500  
ACACCAAAGTATGTGAAATATAGAGATGCTGGTACAGGTATCCGTGAATACAACGATGGA  
ThrProLysTyrValLysTyrArgAspAlaGlyThrGlyIleArgGluTyrAsnAspGly

1510 1520 1530 1540 1550 1560  
ACATTGATATGAAGCGAGACCAAGATTCAACAAGCCAAGTGAAACAAATGCATACAAC  
ThrPheGlyTyrGluAlaArgProArgPheAsnLysProSerGluThrAsnAlaTyrAsn

1570 1580 1590 1600 1610 1620  
GTAACGACAAATCAAGATGGCAGTAACATATGGCGCTCGCCCGACACAAAAAAGCCA  
ValThrThrAsnGlnAspGlyThrValThrTyrGlyAlaArgProThrGlnLysLysPro

1630 1640 1650 1660 1670 1680  
AGCAAAACAAATGCATACAACGTAACAACACATGCAATGGTCAGATATCATATGGCGCT  
SerLysThrAsnAlaTyrAsnValThrThrHisAlaAsnGlyGlnIleSerTyrGlyAla

1690 1700 1710 1720 1730 1740  
CGCCCGACACAAAAAAGCCAAGCAAAACAAATGCATACAACGTAACAACACATGCAAAT  
ArgProThrGlnLysLysProSerLysThrAsnAlaTyrAsnValThrThrHisAlaAsn

1750 1760 1770 1780 1790 1800  
GGTCAAGTATCATATGGCGCTCGCCCGACACAAAAAAGCCAAGCAAAACAAATGCATAT  
GlyGlnValSerTyrGlyAlaArgProThrGlnLysLysProSerLysThrAsnAlaTyr

1810 1820 1830 1840 1850 1860  
AACGTAACAACACATGCAATGGTCAAGTATCATACGGAGCTCGCCCGACATACAAGAAG  
AsnValThrThrHisAlaAsnGlyGlnValSerTyrGlyAlaArgProThrTyrLysLys

1870 1880 1890 1900 1910 1920  
TCAAGCGAAACAAACGCATATAACGTAACAACACATGCAGATGGTACTGCGACATATGGG  
SerSerGluThrAsnAlaTyrAsnValThrThrHisAlaAspGlyThrAlaThrTyrGly

1930  
CCTAGAGTAACAAAATAA  
ProArgValThrLys\*\*\*

	a	b	c	d	e	f	g
1							
11							Met Lys Lys Gln Ile Ile Phe Leu Gly Ala
21							Leu Ala Val Ala Ser Ser Leu Phe Thr Trp
31							Asp Asn Lys Ala Asp Ala Ile Val Thr Lys
41							Asp Tyr Ser Gly Lys Ser Gln Val Gln Lys
51							Lys Glu Arg Gln Asn Ser Ala Ser Ile Ser
58	Ile	Lys	Asn	Leu	Glu	Leu	Asp Ser Tyr Tyr Trp Asp Ile
65	Phe	Thr	Ala	Ala	Leu	Asp	
72	Leu	Glu	Asp	Tyr	Arg	Tyr	
79	Glu	Lys	Glu	Tyr	Glu	Lys	
86	Lys	Asp	Gln	Leu			
90	Met	Thr	Arg	Ile	Leu	Ser	
97	Val	Lys	Tyr	Leu			
101				Leu	Glu	Gln	
105	Ile	Lys	Glu	Tyr	Asp	Lys	
111	Tyr	Lys	Asp	Leu			
115	Tyr	Lys	Glu	Tyr	Met	Ser	
122			Asn	Pro	Thr	Ser	
127	Val	Lys	Arg	Ala	Asn		
132	Phe	Asp	Gln	Tyr	Asn		
137	Ile	Glu	Asp	Leu	Arg	Glu	
144			Glu	Tyr	Asn	Asp	
149	Leu	Ser	Ser	Ile	Lys	Asp	
156	Val	Glu	Thr	Phe	Lys	Ser	
163	Val	Gln	Lys	Ile	Glu	Tyr	
170	Asn	Lys	Glu	Leu	Lys	Ser	
177			Ser	Tyr	Glu	Glu	
182	Lys	Lys	Ala	Ala	Ser	Arg	
188	Val	Asp	Asp	Leu	Ala	Asn	
195	Ala						
196							
206							Tyr Ser Val Tyr Phe Ala Phe Val Arg Asp
216							Thr Gln His Lys Thr Glu Ala Leu Glu Leu
226							Lys Ala Lys Val Asp Leu Val Leu Gly Asp
234							Glu Asp Lys Pro His Arg Ile Ser
237	Ile	Glu	Asn	Glu	Met		
242	Leu	Lys	Asp	Leu	Glu	Ser	
249	Ile	Glu	Asp				
252							
262							Phe Phe Ile Glu Thr Gly Leu Asn Lys Pro
264	Ile	Thr	Ser	Tyr	Asp	Ser	
271	Lys	His	Asp	Tyr	Lys	His	
278	Arg	Glu	Gly	Phe	Glu	Ala	
285	Val	Lys	Glu	Thr	Arg	Glu	
292	Val	Asp	Lys	Ala	Asp	Glu	

Fig. 4

	451		500
DB	LKGtQGESSD IEVKPQATET TEASQYGPRP QFNKTPKYVK YRDAGTGIRE		
8325-4	LKGtQGESSD IEVKPQATET TEASQYGPRP QFNKTPKYVK YRDAGTGIRE		
213	LKGiQGESSD IEVKPQATET TEASQYGPRP QFNKTPKYVK YRDAGTGIRE		
BB	LKGiQGESSD IEVKPQATET TEASQYGPRP QFNKTPKYVK YRDAGTGIRE		
	501		550
DB	YNDGTFGYEA RPRFNKPSET NAYNVTTNQD GTVtYGARPT Q.....		
8325-4	YNDGTFGYEA RPRFNKPSET NAYNVTTthan GqVSYGARPT ykKPSETNAY		
213	YNDGTFGYEA RPRFNKPSET NAYNVTTNQD GTVSYGARPT QNKPSSETNAY		
BB	YNDGTFGYEA RPRFNKPSET NAYNVTTNQD GTVSYGARPT QNKaSETNAY		
	551		600
DB	.....		
8325-4	NVTTHANGQV SYGARPTQ.....		
213	NVTTHANGQV SYGARPTQ.....		
BB	NVTTHANGQV SYGARPTQkk psetnaynvt thangqvsyg arptynkpse		
	601		650
DB	.....		
8325-4	.....kKPSKTNA YNVTTTHANGQ ISYGARPTQk		
213	.....nKPSKTNA YNVTTthNGQ VSYGARqaQn		
BB	.....nKPSKTNA YNVTTTHANGQ VSYGARPTQk		
	tnaynvttchg ngqvsygarp tykKPSKTNA YNVTTTHANGQ VSYGARPTQn		
	651		700
DB	KPSKTNAYNV TTHANGQVSY GARPTqKKPS kTNAYNVTTTH ANGQVSYGAR		
8325-4	KPSKTNAYNV TTHANGQVSY GARPTy....		
213	KPSKTNAYNV TTHANGQVSY GARPTyKKPS ETNAYNVTTTH ANGQVSYGAR		
BB	KPSetNAYNV TTHANGQVSY GARPTqnKPS ETNAYNVTTTH gNGQVSYGAR		
	701		730
DB	PTYKKsSetN AYNVTTHADG TATYGPRVTK		
8325-4	...KKPSkTN AYNVTTHADG TATYGPRVTK		
213	PTqKKPSeTN AYNVTTHADG TATYGPRVTK		
BB	PTYnKPSkTN AYNVTTHADG TATYGPRVTK		

Fig. 5

## Strains

	1		50
DB	MKKQIIIFLGA LAVASSLFTW DNKADAIIVTK DYSGkSqVqk KerqNsasIS		
8325-4	MKKQIIISLGA LAVASSLFTW DNKADAIIVTK DYSGkSqVNa gSK.NGtLid		
213	MKKQIIISLGA LAVASSLFTW DNKADAIIVTK DYSGkSqVNa gSK.NGtLid		
BB	MKKQIIISLGA LAVASSLFTW DNKADAIIVTK DYSGkSqVNa gSK.NGtLid		
	51		100
DB	DsYyWdIikn LEIQftaAld LLEdYrYGEK eYekAKDqLM TRILsEvkYL		
8325-4	sRYlnsalyY LEdyiiYAg LcnkYeYGDn iYKEAKDRLL ekVLrEDQYL		
213	DYYyWkIids LEaQftgAid LLEnYkYGDp iYKEAKDRLL TRVLGEDQYL		
BB	DWYlkgrlts LESQFinAld iLEtYhYGEK eYKDAKDkLM TRILGEDQYL		
	101		150
DB	LEqKikeyDk YKdLYKeYms kNPTSK.vKr AnFdqYNiED LreKEYNDLL		
8325-4	LERKksqYED YKqWYanYKk ENP.rtdLKM AnFhKYNLEE LsMKEYNELq		
213	LkkKideYEl YKKWYKssnK nt.....nM ltFhKYNlyn LTMnEYNDiF		
BB	LERKkvqYEE YKKLYqkYKe ENPTSKgLKL ktFdqYtiED LTMrEYNELt		
	151		200
DB	sSiKDAVetF ksDVqkIEye NkELKsYsyE EEKKAasrVd DLankaysvy		
8325-4	DaLKrAlDDF hrEVKdIkdk NsDLKtFnaa EEDKATkeVy DLvseidtlv		
213	nSLKDAVYqF nKEVKeIEhK NvDLKqFdKd gEDKATkeVy DLvseidtlv		
BB	ESLKSaVkdF eKDVeKIEng hhdLKpFtdE mEEKATsrVd DLankaysvy		
	201		250
DB	fAFVRDtqhk teALELkAKv DLVLGDeDkP HrIsNkRIen EMikDLeSII		
8325-4	vsYyGdkdyg ehAkELrAKI DLILGDtDnP HkItNERIKK EMidDLnSII		
213	vtYyaDkdyg ehAkELrAKI DLILGDtDnP HkItNERIKK EMidDLnSII		
BB	fAFVRDtqhk teALELkAKv DLVLGDeDkP HrIsNERIEk EMikDLeSII		
	251		300
DB	EDFFiETgln kPdnITSYDs sKHdYKkHRE ...gFEaLVk ETreAVdkAD		
8325-4	DDFFMETKqN rPKsITkYnp tCHNYKtNSD NKPNFDkLve ETkkAVKEAD		
213	DDFFMETKqN rPnsITkYDp tKHNFKEKSE NKPNFDkLve ETkkAVKEAD		
BB	EDFFiETgln kPgnITSYDs sKHhYKkHSE ...gFEaLVk ETreAVanAD		
	301		350
DB	ESWKTkTVKt YGEaETKahV VKEEKkVEEP QAPKVGnQQG dKTTvdKeVE		
8325-4	DSWKTkTVKk YGETETKSPV VKEEKkVEEP QAPKVDnQQE VKTTAGKAEE		
213	ESWKTkTVKk YeETvTKSPV VKEEKkVEEP QlPKVGnQQE VKTTAGKAEE		
BB	ESWKTkTVKk YGEsETKSPV VKEEnKVEDP QsPKfdnQQE VKTTAGKAEE		
	351		400
DB	aTQPVAQhLV gIPQGTITGE IVKGPDYPTM ENKmLQGEIV QGPDFLTMEQ		
8325-4	TTQPVAQPLV KIPQGTITGE IVKGPEYPTM ENKtVQGEIV QGPDFLTMEQ		
213	TTQPVAQPLV KIPQeTiYGE eVKGPEYPTM ENKTLQGEIV QGPDFLTMEQ		
BB	TTQPVAQPLV KIPQGTITGE IVKGPEYPTM ENKTLQGEIV QGPDFLTMEQ		
	401		450
DB	nrPSLSdNYT QPsvclpsic gestPtNPIL EGLEGSSSKL EIKPQGTEST		
8325-4	sgPSLSnNYT nP..... .pltnPIL EGLEGSSSKL EIKPQGTEST		
213	nrPSLSdNYT QP..... .TTpNPIL EGLEGSSSKL EIKPQGTEST		
BB	sgPSLSdNYT QP..... .TTpNPIL EGLEGSSSKL EIKPQGTEST		